THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
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Application for Research Grant
(Use extra pages as needed)

Date Apr. 20,1974

1. Principal Investigator (give title and degrees): George M. Hass, M.D., Acting Chairman, Department of Pathology

2. Institution & address: Rush-Presbyterian-St. Luke's Medical Center
1753 W. Congress Parkway
Chicago, Illinois 60612

3. Department(s) where research will be done or collaboration provided: Department of Pathology

4. Short title of study: Pathogenesis of Cholesterol-Vitamin D-Nicotine Induced Arteriosclerosis

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims: The first purpose is to further establish relations between arterial disease induced by cholesterol-vitamin D-nicotine regimens and the following factors: (1) adrenocortical function, (2) hepatocellular function and (3) catecholamine function.

The second purpose is to determine whether the vasculotoxic action of nicotine in combination with modest amounts of dietary cholesterol and vitamin D can be minimized by elimination or blockade of the pathway by which enhancement of the vasculotoxic action occurs.

These aims are based principally on our past and current observations. Some have been published in detail (see Paragraph 13). Others are in manuscript form (see attached manuscript). Still others are under continuing study.

The most relevant observations have been obtained during the period of support by the Committee for Research on Tobacco and Health, AMA Research and Education Foundation. The funding by this Committee was terminated as of 02-01-74, though we have been allowed to use a small unexpended sum during the period, 02-01-74 to 02-01-75.

The content of our last Progress Report to the above Committee is as follows and summarizes the reasons for the current direction of research and our present proposal.

Progress Report

In this report the progress since the beginning of this study will be summarized and brought up to 10-25-72, the date of this writing.

First: Chronic sublethal injections of nicotine over a period of several months in more than 100 rabbits did not produce any arterial or other disease, recognizable microscopically.

Second: In the aforementioned series of animals, nicotine had no sustained action on the levels of serum cholesterol.

Third: When daily injections of nicotine in mineral oil were given subcutaneously to rabbits maintained on a specific regimen of dietary cholesterol and subcutaneous injections of vitamin D in corn oil, a severe arterial disease developed. The regimen in the absence of nicotine was not productive of this disease. The disease usually occurred within 2-4 months and was characterized principally by peripheral calcific arteriosclerosis, intimal proliferation, atheromatous deposition and thromboarteritis.

Fourth: A controlled study of this disease by gross and detailed microscopic methods in more than 150 rabbits led to the following conclusions. First, it was apparent that all animals did not react equally but that 30 to 75 percent of those on the complete multifactorial regimen developed the disease. Second, nicotine enhanced the calcifying action of vitamin D in some unexplained way. Third, the calcification of arterial walls stimulated proliferation of the intima to an extent about equal to the thickness of arterial media affected by calcium deposition. Fourth, the loci of intimal proliferation became sites for elective accumulation of plasma lipids, even though the cholesterol levels were much lower than those ordinarily required for significant lipid accumulation. Finally, these reactive sites, especially in peripheral arteries of the skeletal muscle, ear and duodenum frequently (about 30-50 percent) were involved by arteritis and thrombosis. There was more than a passing resemblance of this disease to peripheral calcific arteriosclerosis with thrombotic complications in man.

Fifth: Peripheral calcific thromboarteritis of the type induced by nicotine was seldom noted in animals on the cholesterol-vitamin D regimen without nicotine. This fact led to a series of studies directed toward an analysis of the means by which nicotine produced the observed arterial disease. These studies involved an inquiry into lipid metabolism, adrenal function and liver function.

Sixth: Two factors pertinent to lipid metabolism were evaluated. One factor was the level of serum cholesterol. It was soon clear that the overall chronic persistent result of nicotine was to slightly depress the level of serum cholesterol. Nicotine, however, had a much more impressive effect on the levels of plasma free fatty acids (FFA). After a long series of studies with repeated determinations of plasma FFA at monthly intervals, it was concluded that rabbits could be divided into three groups. Those that had the greatest nicotine-induced rise in plasma FFA tended to develop calcific arteriosclerosis complicated by thrombosis. Those that had a modest rise in plasma FFA following injections of nicotine tended to develop calcific arteriosclerosis alone. The few animals that

had a negligible rise in plasma FFA tended to have no peripheral arterial disease. It would be of great interest if a similar analysis could be made by clinical investigators concerned with "risk factors" in human arteriosclerosis.

Seventh: We followed the lead obtained by plasma FFA analyses with the thought that the increased level of plasma FFA induced by nicotine might be responsible for the tendency to thrombosis. ACTH and heparin, both of which produced far greater rises of plasma FFA than nicotine, were given to a series of animals on the cholesterol-vitamin D regimen with and without nicotine. There was no effect of ACTH or heparin on the arterial disease. Hence, it was concluded that the magnitude of rise of plasma FFA induced by nicotine was merely an indicator of the probability of occurrence of nicotine-induced arteriopathy and not an essential factor in pathogenesis of the arterial disease.

Eighth: Attention was next directed to the role of the adrenal gland in mediation of the action of nicotine. With the assumption that the observed effects of nicotine were adrenergic, a series of rabbits was given multiple daily injections of large amounts of adrenalin in oil for several weeks. Adrenalin did not duplicate the action of nicotine. A second series of rabbits was given reserpine in sub-lethal amounts to block the theoretical catecholamine effects of nicotine, both at the adrenal medullary and peripheral storage levels. Reserpinized animals developed severe arterial disease, much more so in the duodenal than peripheral arterial systems. This is now being restudied because of the indication that we may have struck upon a means of evaluating factors which regulate the vagaries of distribution of arteriosclerosis in man, as well as in the experimental animals.

Ninth: Inasmuch as we could not duplicate the action of nicotine by use of adrenalin or block its action by reserpine, we resorted to adrenalectomy - a difficult procedure in the rabbit. It was concluded from a series of 50 adrenal-ectomized rabbits that, if the amount of residual and regenerated adrenal tissue was more than 200 milligrams and less than about 500 milligrams, the incidence of calcific arteriosclerosis and thrombosis was greatly reduced among animals on the cholesterol-vitamin D-nicotine regimen. If the amount of residual and regenerated adrenal tissue was less than 200 milligrams (normal 1200 milligrams), the occurrence of arterial disease (1/19) was practically prevented. Current studies are directed toward evaluation of the relative importance of adrenal cortex and medulla in the mediation of the arterial disease.

Tenth: It is well known that the liver has a central role in the metabolism of catecholamines, vitamin D and substances such as nicotine. We have shown that among more than 55 rabbits surviving in good health for 6-12 months with severe progressive nodular cirrhosis, tolerance to vitamin D was reduced 50 to 100-fold. Hence, the action of nicotine was simulated by carbon tetrachloride-induced cirrhosis. However, the effects of cirrhosis and nicotine were not additive as we had reason to suspect in theory. Our present studies are directed toward further evaluation of the role of the liver in mediating the effects of nicotine and vitamin D. This is relevant to man for there is evidence that cirrhosis and cigarette-smoking seem to be synergistic as "risk factors" in arteriosclerosis.

Eleventh: Our current and projected studies are directed toward an explanation of the undesirable effects of nicotine in the multifactorial cholesterol-vitamin D-nicotine system. There is no doubt that nicotine enhances the vasculotoxic action of vitamin D and there is more than a little evidence that excessive cigarette smoking may be associated with excess arterial calcification in man. It is also generally agreed that vitamin D or its metabolites have much to do with calcification in man. If nicotine acts as a stress factor and if its undesirable action in man is related to individual reactions to stress, our attention to the adrenal and the liver as mediators of reactivity seems justified. Reduction of adrenal function by surgical ablation minimized nicotine action but it remains for us to determine whether this was due to reduction of medullary or cortical function or both. We suspect both though we have been unable to implicate medullary function either by use of exogenous adrenalin or reserpine. To what extent an interference in liver function was involved remains a puzzle. There was a similarity between the "activation" of vitamin D, not only by nicotine but also by reduced liver function in the cirrhotic animal. It would seem advisable, therefore, for us to give increasing attention to nicotine enhancement of arterial calcification and the conditions extent to which the regulation of calcium metabolism by mineralocorticoids, vitamin D and catecholamines or similar neurohumoral agents is involved. Our results with adrenalectomy were adequate proof that arterial calcification and subsequent thrombosis can be minimized and prevented without undue impairment of the health of the animal. If the mechanisms implicated in this prevention can be understood, prevention of calcification of arteries in man may be realized. If this can be achieved, arteriosclerosis and its complications in man can be practically eliminated. In proceeding to this end in the projected studies, a systematic study of the reasons for the susceptibility of the cirrhotic animal to arterial calcification and the resistance of the adrenalectomized animal to the same disease is being undertaken using the nicotine-cholesterol-vitamin D regimen. In carrying out this study primary attention will be given to the detection of active metabolites of vitamin D in the plasma, the binding of these metabolites to plasma proteins and the elective binding of these metabolites to receptor sites affected by nicotine.

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mediation of the arterial disease.

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8. Brief statement of working hypothesis:

There is evidence that excessive cigarette smoking is associated with an increased incidence of severe arteriosclerosis in some people. The nature of this association is not clear but it is assumed that the sympathomimetic action of nicotine is responsible. Such an assumption might well take into account the possibility that nicotine enhances the arteriosclerotic action of genetic and other "risk factors," which govern the pathogenesis of the disease. Inasmuch as we now have some experimental proof for this, as summarized in the Progress Report in paragraph 7, it becomes desirable, first to define the metabolic pathway of enhancement and then to seek the means to eliminate it.

9. Details of experimental design and procedures (append extra pages as necessary) i.

a. Our experimental approach depends principally on use of an animal model system which has disclosed a pathogenetic link between use of nicotine and development of severe calcific atheroarteriosclerosis complicated by thrombosis in rabbits given modest amounts of vitamin D and dietary cholesterol.

The basic regimen is as follows. Male New Zealand random-bred albino rabbits, about 4-6 months old and weighing 6-8 pounds will be used. They will be fed a diet containing sufficient cholesterol and corn oil to maintain the average serum cholesterol levels between 300 and 400 milligrams percent, at which concentration in the absence of other vasculotoxic factors, no significant atherosclerosis develops. The second "risk factor" of the regimen will be subcutaneous injection of vitamin D (Ergocalciferol) in corn oil (100,000 IU per ml) in a dosage of 25,000 IU to 100,000 IU twice every fourth week. The third "risk factor" will be nicotine, as the base (100 mgm/ml in mineral oil), given intramuscularly five days each week. The initial dose will be 20 milligrams and this will be increased 2 milligrams each week up to a maximum tolerable dose, usually less than 50 milligrams. Each experiment will vary in duration but most of them, with good fortune, will last for an average of 20-30 weeks. At the end of each experiment, complete autopsies will be done and a thorough microscopic study made of the affected organs and arterial systems (40-60 paraffin sections stained with hematoxylin and eosin). Special stains will be used when indicated.

The principal routine analytical studies will be serum cholesterol, serum calcium, and serum phosphate determinations every two months, or more often when indicated.

b. The variable of hyperlipemic hypercholesteremia

We do not intend to concentrate on this variable except insofar as dietary regulation will be used to maintain the serum cholesterol levels in the usual high adult human range where it seems to be most significant as a serious "risk factor." This may not always be possible because of the variable effects of different regimens on the health and eating habits of the animals but the average levels will not exceed 350 milligrams percent.

c. The variable of plasma FFA

Our previous studies have shown that animals with the greatest nicotine-induced rise of plasma FFA were most likely to develop severe calcific arterio-sclerotic thromboarteritis. We do not plan to explore this "genetic matter" further because ACTH or heparin, both of which induce far greater increases in plasma FFA than nicotine, did not influence the development of atheroarteriosclerosis or thrombosis. The "genetic matter" involves something more than the simple increase in plasma FFA.

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(see continuation sheet)

9. (continuation)

The effect of adrenalectomy on the nicotine-induced rise of plasma FFA may be studied later, if time permits. Such quantitative study requires many repeated successive spaced analyses and many controls which do not seem as relevant at this time as the proposed experiments.

d. The variable of vitamin D

It seems clear from results of our studies, to date, that the vasculotoxic effects of nicotine are mediated principally through the action of vitamin D. Nicotine enhances the calcifying action of vitamin D and in some obscure way induces a thromboarteritis in certain distal peripheral arterial systems. To some extent this also seems to be an effect of nicotine in some people. The statistical evidence indicates that heavy cigarette smoking seems often to be associated with excessive calcification of coronary arteries. It is also well known that the only useful treatment of thromboangiitis obliterans in man is to stop smoking. In our projected experiments we will use ranges of dosage of vitamin D that best bring out the vasculotoxic synergism of the nicotine-vitamin D-cholesterol regimen. For study of nicotine enhancement of the calcifying arteriosclerotic action of vitamin D, the dosage of 25,000 to 50,000 IU of vitamin D twice every fourth week is most useful. For study of enhancement of action of nicotine in production of thromboarteritis, a dosage of 50,000 to 100,000 IU twice every fourth week is the best. It is likely, judging from experience thus far, that the optimal dosage for production of vasculotoxic effects in cirrhotic animals may be no more than 1,000 IU while that in totally adrenalectomized animals may exceed 1,000,000 IU. In any event, the quantitation of relations between "risk factors" and impairment of adrenal or hepatic function will be essential if we are to draw adequate conclusions.

e. The variable of nicotine

In our first studies several years ago, nicotine was added to the diet. This proved to be unsatisfactory because of individual dietary habits. Later, nicotine was given subcutaneously in several rapidly absorbed vehicles. This led to erratic results. Finally, nicotine was dissolved in mineral oil (White Oil #35 USP, American Oil Co.) and given intramuscularly. This provided the desired degree of slow absorption and reduced the frequency of grand mal seizures. These seizures ordinarily occurred immediately after injection and were more common as the dosage was increased. However, some animals tended to react repeatedly at low dosages while others failed to react at all despite use of very high dosages. Our results indicate that the optimal dosage for obtaining the vasculotoxic effects among animals on the cholesterol-vitamin D-nicotine regimen is about 20-30 milligrams per day, though statistically significant effects have been obtained at dosages as little as 2-5 milligrams of nicotine given intramuscularly in mineral oil, no more than twice each week. In our projected studies, we plan to use the maximum effective dosages within the limits of the tolerance of the animals. Whether the limit of tolerance, as manifest by grand mal seizures, is related to the limit of tolerance to vasculotoxic effects is to be evaluated. It seems likely that this dosage may be very high in adrenalectomized animals and much lower in animals with cirrhosis or animals given reserpine.

f. The variable of adrenocortical function

It is at the level of this variable where some insight may be gained as to the pathway of mediation of the vasculotoxic action of the cholesterol-vitamin D-nicotine regimen. Analysis of the role of adrenocortical function would be simplified if there was independence of adrenal cortical and adrenal medullary activity. Unfortunately, this is not so because recent evidence strongly supports the view, long held by students of Addison's Disease, that adrenal medullary enzymatic conversion of norepinephrine to epinephrine is regulated by adrenal glucocorticoids. It has been shown that in the absence of the hypophysis, this regulation can be taken over by administration of glucocorticoids or ACTH.

It is technically difficult, if not impossible, to demedullate the adrenals

of the rabbit without serious impairment of cortical tissue. It is possible, however, to do a complete adrenalectomy and thereby eliminate adrenal medullary tissue, unless recognized "accessory" medullary tissue remains. In our experience, if adrenalectomy is incomplete and corticosteroid therapy not used, there is a conspicuous regeneration of residual adrenal cortex, which could be interpreted as hyperplastic "accessory adrenals." If a complete adrenalectomy is done, the animal will not survive unless corticosteroid therapy is instituted. Under these conditions in our experience so-called "accessory" adrenal tissue is absent or insignificant except in rare instances. [illegible] this a[illegible]. Complete adrenalectomy is best done as follows. The right adrenal is first removed by intracapsular enucleation and ablation with silver nitrate. One week later the left adrenal is surgically excised after ligation of its blood supply. The glucocorticoid, Dexamethasone (1.0 milligram) and the mineralo-corticoid, desoxy-corticosterone (0.5 milligrams) are then injected subcutaneously, daily, five days each week. After four weeks, the animals are placed on a diet containing 250-500 milligrams percent of cholesterol and administration of nicotine and vitamin D instituted. For the sake of completeness, appropriate control paired experiments will be done using animals with intact adrenals on such variations of the cholesterol-vitamin D-nicotine regimen as those being assessed in the adrenalectomized pair. We already have data using sham or non-adrenalectomized animals so that most studies will be made on fully adrenalectomized animals or at least on animals with little prospect of terminal adrenal tissue weighing more than 150 milligrams (about one-tenth the amount present in controls on the same regimen).

We expect, based upon current experience, that when there is less than about 150 milligrams of adrenal tissue at termination following adrenalectomy (usually 15-35 weeks), the regimen should have no significant vasculotoxic or thromboarteritic action. If more than about 250-350 milligrams is present at termination, some degree of vasculotoxic calcific but little or no thromboarteritis action should persist. In both instances the amount of microscopically demonstrable residual medulla will be negligible and the maintenance of good health will depend to a large extent on adequate corticosteroid therapy. The principal question to be answered, therefore, is how a small demedullated remnant of adrenal cortex supports the vasculotoxic action of the cholesterol-vitamin D-nicotine regimen. This would seem to exclude a role for epinephrine and medullary norepinephrine. It may be assumed, in the presence of adequate steroid substitution therapy, that an intrinsic essential product of cortical cell function is not involved. An alternative assumption is that support for the vasculotoxic action is provided by an extrinsic product modified by some adrenocortical enzyme system.

g. The variable of hepatic function

Our recent studies have shown that the standard regimen developed for production of cirrhosis by use of carbon-tetrachloride in the rabbit also produces an extreme sensitivity to vitamin D. This is often so pronounced that generalized arterial calcification occurs at a dosage of vitamin D less than 1-2 percent of the amount required to produce the same disease in control animals.

This proposed program, therefore, involves a continuation of these studies of the role of impaired hepatic function. The following procedure will be used.

Carbon tetrachloride (CP) will be dissolved in mineral oil (White Oil #35 USP, American Oil Co.) to give a ten percent solution. This will be given subcutaneously at a 3-day interval, twice each week for eight weeks and thereafter, twice every other week. The dosage will be 0.3 milliliter of the 10 percent solution (0.03 milliliter of carbon tetrachloride) per pound of body weight. At the end of the fourth week of the regimen, animals will be placed upon various permutations of the cholesterol-vitamin D-nicotine regimen. Addition of cholesterol will be 250-500 milligrams per 100 grams of diet. Dosage of vitamin D will be varied from 0 to 1580 IU twice every fourth week. Dosage of nicotine will be varied from the standard amount to zero. Appropriate control animals will be placed on the same regimen first without use of carbon tetrachloride. We shall also seek a minimally effective hepatotoxic dosage schedule so as to evaluate the action of carbon tetrachloride at dosages insufficient for production of cirrhosis.

A second method for production of cirrhosis in the rabbits needs to be developed so as to distinguish the effects of cirrhosis and impaired hepatic function from those of carbon tetrachloride which has a specific action as a lipid peroxidant. The first substance to be tried will be thioacetamide. This is a controllable hepatotoxic reagent for inducing cirrhosis in the rabbit without causing too many undesirable extrahepatic effects.

In each instance the animals on the hepatotoxic regimens will be kept for periods of 6 to 12 months during which time relations between impaired hepatic function, cirrhosis and vasculotoxicity of the cholesterol-vitamin D-nicotine regimen can be assessed.

h. The variable of the hepato-adrenal axis

We have shown that there are reasons for believing that mediation of the vasculotoxic effects of the cholesterol-vitamin D-nicotine regimen involves the hepato-adrenal axis. One reason is that total adrenalectomy practically eliminates the vasculotoxic action of the regimen, even though effective corticosteroid therapy is used to sustain the health of the animals. The second reason is that the vasculotoxicity of the regimen is greatly enhanced in animals given sufficient carbon tetrachloride to produce severe nodular cirrhosis. In many ways the effects of the cirrhotic state mimic the effects of nicotine because cirrhosis in the absence of nicotine enhances the action of vitamin D and favors occurrence of thromboarteritis. epinephri In order to inquire into the antagonism between the effects of total adrenalectomy and those of cirrhosis in mediation of the vasculotoxic action of the cholesterol-vitamin D-nicotine regimen, we propose to assess the effect of combining adrenalectomy with induced cirrhosis in the same animal. Thus far, this has been accomplished in our laboratory by institution of the carbon tetrachloride regimen in adrenalectomized animals. All animals with cirrhosis subjected to adrenalectomy have, thus far, due to most unusual complications failed to survive longer than a few days. From our viewpoint, however, a persistent examination of the respective roles of impaired adrenal and hepatic function is essential to an understanding of the pathogenesis of calcific atheroarteriosclerosis with thromboarteritis induced by the cholesterol-vitamin D-nicotine regimen.

i. The variable of catecholamine action

Nicotine is a sympathomimetic reagent and has many actions which resemble those of the catecholamines. It is known that adrenalectomy eliminates the rise of plasma FFA due to the use of nicotine. It is also known that certain effects of nicotine may be minimized or eliminated by use of reagents that block adrenergic receptors. The pharmacology of the subject remains complex, and to some extent controversial, because of differences in reaction of different species and different parts of the vascular system to different dosages of nicotine.

We propose to approach the subject by use of reserpine. The reason for use of this drug is based upon its principal peripheral pharmacologic action. It is generally accepted that reserpine blocks the uptake of norepinephrine into adrenergic sympathetic nerve terminals and thereby exposes norepinephrine to monoamine oxidase inactivation. The overall effect is a reduction in the supply of the neurotransmitter delivered to the receptors. There are a good many theories among the explanations for reserpine action but there is no doubt that it exerts a profound depressant action in prolonged experiments with rabbits on appropriate dosage schedules.

It is our purpose, therefore, to study effects of chronic reserpinization in rabbits on the cholesterol-vitamin D-nicotine regimen. A dosage of 0.35 to 0.50 milligrams intramuscularly three times each week seems effective and practical. In current pilot experiments the lower dosage of reserpine induces an extreme sensitivity to nicotine. This is often impressive, individually characteristic and contrary to expectation although pharmacologists can most likely give an adequate explanation. Perhaps, this is a manifestation of "supersensitivity." It may be that a relation between the sensitivity to nicotine and the vasculotoxicity of the cholesterol-vitamin D-nicotine regimen may be found in the general area of catecholamine function and adrenergic receptors. Whether these observations on reserpinized animals will provide some insight into the action of nicotine on the central nervous system and the neural pathway of hepato-adrenal axis mediation of its vasculotoxic effects may not be unduly speculative.

j. Schedule of proposed experiments

The following schedule and distribution of experimental animals is proposed with each group being kept at about the indicated number.

1. 5 adrenalectomized rabbits will be placed on the standard DOCA-Decadron-cholesterol-vitamin D (100,000 IU)-nicotine (20 milligrams) regimen for a maximum of 40 weeks.

2. 5 adrenalectomized rabbits will be placed, postoperatively, on the DOCA-Decadron-cholesterol-vitamin D (1580 IU)-nicotine (20 milligrams)-carbon tetrachloride regimen for a maximum of 40 weeks.

3. 5 rabbits will be placed on the standard carbon tetrachloride regimen for twenty weeks. Following adrenalectomy they will be maintained on the standard DOCA-Decadron-cholesterol-vitamin D (100,000 IU)-nicotine (20 milligrams) regimen for up to 40 weeks.

4. 5 rabbits will be placed on the standard carbon tetrachloride regimen for 20 weeks. Thereafter, they will be kept on the standard DOCA-Decadron-cholesterol-vitamin D (100,000 IU)-nicotine (20 milligrams) regimen for up to 40 weeks.

5. 5 rabbits will be placed on the standard reserpine (0.35-0.50 milligrams)-cholesterol-vitamin D (100,000 IU -nicotine (20 milligrams) regimen for up to 40 weeks.

6. 5 rabbits will be placed on hepatotoxic chronic dosage of thioacetamide administered, intramuscularly, with the intent of producing a chronic cirrhosis comparable with that induced by the carbon tetrachloride regimen.

7. 5 rabbits will be kept on the standard DOCA-Decadron-cholesterol-vitamin D (100,000 IU)-nicotine (20 milligrams) regimen for up to 40 weeks as a control group.

8. 5 rabbits will be placed on the standard DOCA-Decadron-cholesterol-vitamin D (1580 IU)-nicotine (20 milligrams) regimen for up to 40 weeks.

9. Other required control groups have either been studied in the recent past or are currently under study together with some of the experimental groups. Though the numbers of animals are not as many as we would like, the experiments are

9. (continuation, 5)

essentially of a pilot type to be enlarged upon or modified as data accumulate from the study of animals that do not survive more than a few weeks. It is to be remembered that vasculotoxicity of the standard regimen is usually conspicuous within 12 weeks at a total vitamin D dosage of 600,000 IU. However, the mortality rates may be such that the total number of animals on experiment at any time will seldom exceed 40. Otherwise, the expense involved in their maintenance in the Animal Resources Facility at 80 cents each per day (total estimate of $32.00 per day) becomes too great.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

One histology technical laboratory is used full-time in this study.

One special laboratory of 300 square feet is used exclusively for records, files, autopsy performance and specimen storage.

Eight special research analytical laboratories contain required instruments as follows: Beckman Amino Acid Analyzer, Perkin-Elmer Atomic Absorption Spectrophotometer, DU and DK Spectrophotometers, Spinco Ultracentrifuges, Freeze-Dry Equipment, Deep-Freeze Equipment, Dual Gas Chromatographs, RCA and Phillips EM 300 electron microscopes and appropriate analytical balances. These are available for the proposed and other relevant analytical determinations.

Animal Hospital facilities for the housing and care of as many as 60 rabbits are available.

A general diet preparation kitchen is available and in use for preparation of the cholesterol diets.

A surgical operating suite required for adrenalectomy is available two mornings each week.

11. Additional facilities required:

No additional facilities are required.

12. Biographical sketches of investigator(s) and other professional personnel (append):

(see attached sheets)

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

(see attached sheets)

Curriculum Vitae

George Marvin Hass, M.D.

Title: Professor of Pathology, Chairman, Department of Pathology

Birthdate: REDACTED

Place of Birth:

Education: University of Iowa (B.S., 1927), Harvard University (M.D., 1929)

Honors: Junior Fellow, Society of Fellows, Harvard Univ.

Major Research Interest: Experimental and human pathology, arteriosclerosis, growth, regeneration, neoplasia

Research Support: Human and Experimental Atheroarteriosclerosis, USPHS Grant HE-03215, #35,300 (1971-72), $35,300 (1972-73);
Pathologic Effects of Lead, USPHS Grant NB-04872, $20,600 (1971-72), $20,600 (1972-73);
2-AAF Induction of Urinary Bladder Tumors, USPHS Grant CA-08857, $21,460 (1971-72), $21,460 (1972-73);
Lymphoma and Leukemia, USPHS Grant CA-11437, $24,700 (1972-73);
Miscellaneous private and institutional (Other research), $65,000 (1972-73)

Research and/or Professional Experience: Chairman, Department of Pathology, Presbyterian-St. Luke's Hospital, 1946-; Professor of Pathology, Rush Medical College, 1971-; Professor of Pathology, University of Illinois College of Medicine, 1946-1971; Director, USPHS Training Program in Experimental Pathology (NHI, 1949-1957; NIGMS, 1957-1968) (1968-1971); Consultant, Surgeon General, USPHS, 1949-1962; Chief, Dept. of Pathology, USAF School of Aviation Medicine, 1943-1946; Chief Consultant in Pathology, Surgeon General, USAF, 1946-1949; Chief Pathologist, USAF, MC, AUS, 1942-1946; Asst. Professor of Pathology, Cornell Univ., 1939-1942; Instructor to Associate in Pathology, Harvard Univ., 1932-1939; Junior Fellow, Society of Fellows, Harvard Univ., 1934-1937; Resident in Pathology, Peter Bent Brigham Hospital, Boston, 1932-1934; Resident in Pathology, Children's Hospital, Boston, 1931-1932; Interne in Pathology, Peter Bent Brigham Hospital, Boston, 1930-1931; Research Fellow, Physiology, Harvard University, 1929-1930

Selected Relevant References

1. Hass, George M., Trueheart, Richard E. and Hemmens, Anne
Experimental atheroarteriosclerosis due to calcific medial degeneration and hypercholesteremia.
Am. J. Path., 38:289-323, 1961

2. Hass, George M.
Metabolic and Nutritional Factors in Peripheral Vascular Disease.
Chapter 8. In: The Peripheral Blood Vessels. International Academy of Pathology, Monograph No. 4, The Williams and Wilkins Company, 1963, pp. 157-204

3. Hass, George M., Landerholm, Wayne and Hemmens, Anne
Production of calcific atheroarteriosclerosis and thromboarteritis with nicotine, vitamin D and dietary cholesterol.
Am. J. Path., 49:739-771, 1966

4. Hass, George M., Henson, Donald E., Scott, Richard A., McClain, Eldon C. and Hemmens, Anne
Influence of cirrhosis on production of atheroarteriosclerosis and thromboarteritis with vitamin D and dietary cholesterol.
Am. J. Path., 57:405-429, 1969

5. Scott, Richard A., Henson, Donald E., Lesak, Anne, Turner, Robert J., Malikova, Stanislava and Hass, George M.
Relations between metabolic increase of plasma free fatty acids and the occurrence of arteriosclerotic thromboarteritis in rabbits.
Am. J. Path., 70:209-233, 1973

Chief Pathologist, USAF, MC, AUS, 1942-1946; Asst.

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Curriculum Vitae

Reuben Eisenstein, M.D.

Title:	Attending Pathologist, Presbyterian-St. Luke's Hospital and Professor of Pathology, Rush Medical College
Place of Birth:	REDACTED
Birthdate:	REDACTED
Education:	Tulane University (B.S., 1949); Louisiana State University (M.D., 1953)
Honors:	Alpha Omega Alpha
Major Research Interest:	Connective Tissue Disorders
Research Support:	USPHS grant HL-14609, 1972-1975 ($29,849 annually), "The Organization of Arterial Ground Substance." Chicago Heart Association ($10,450 annually, 1973-1974), "A Proteinase Inhibitor in Vascular Walls."
Research and/or Professional Experience:	Professor, Pathology, Rush Medical College, 1971-; Professor, Pathology, University of Illinois, 1969-1971; Assoc. Prof. Pathology, Univ. of Illinois, 1965-1969; Asst. Prof. Pathology, Univ. of Illinois, 1960-1965; Assoc. Att. Pathologist, Presbyterian-St. Luke's Hospital, 1963-; Asst. Attending Pathologist, Presbyterian-St. Luke's Hospital, 1960-1963; Resident, Pathology, Presbyterian-St. Luke's Hospital, 1957-1960; Resident, Pathology, Mt. Sinai Hospital, 1954-1955; Interne, Mt. Sinai Hospital, New York, 1953-1954. Member, Cardiovascular B Study Section, NIH, 1967-1971

Recent Relevant Publications

1. Eisenstein, R., Ellis, H. and Rosato, J.
In vitro studies of vitamin D induced aortic calcification.
Proc. Soc. Exp. Biol. and Med., 132:58, 1969

2. Eisenstein, R., Scott, R.A. and Lesak, A.
Altered lipid binding by mineralized aortic elastin.
Arch. Path., 92:301-306, 1971

3. Eisenstein, R.
Pathological Calcification.
In: The Biochemistry and Physiology of Bone, ed. G.H.Bourne, Academic Press, 1972, Vol. 2, p. 357

4. Eisenstein, R., Arsenis, C., Sorgente, N. and Kuettner, K.E.
Effect of vitamin D on serum and tissue lysozyme.
AMA Arch. Path., 92:301, 1971

5. Eisenstein, R., Larsson, S., Sorgente, N. and Kuettner, K.E.
Collagen-proteoglycan relationships in epiphyseal cartilage.
Am. J. Path., 73:443-452, 1973

Curriculum Vitae

Robert E. Lee, Jr., M.D., Ph.D.

Title: Assistant Attending Pathologist, Presbyterian-St. Luke's Hospital, Assistant Professor of Pathology, Rush Medical College

Birthdate: REDACTED

Place of Birth:

Education: St. Mary's College, Winona, Minn. (B.S., 1954); Loyola Univ. Graduate School (M.S., Anatomy, 1958), Loyola Univ. Stritch School of Med. (Ph.D., Anatomy, 1962); Stritch School of Med., (M.D., 1965)

Research Support: Schweppe Fellowship, $10,000 (1973-74)

Research and/or Professional Experience: Assistant Attending Pathologist, Presbyterian-St. Luke's Hospital and Assistant Professor of Pathology, Rush Medical College, 1972-; Attending Pathologist, Walter Reed General Hospital, 1970-1972; Schweppe Foundation Fellow, Presbyterian-St. Luke's Hospital, 1969-1970 and 1973-1975; Resident, Pathology, Presbyterian-St. Luke's Hospital, 1966-1970; Clinical Fellow, Pathology, American Cancer Society, Presbyterian-St. Luke's Hospital, 1967-1969; Intern, Pathology, Presbyterian-St. Luke's Hospital, 1965-1966; Research Fellow, Loyola Univ., 1962-1965; Royal E. Cabell Fellow, Loyola Univ., 1960-1961; Instr. Pathology, Univ. of Illinois, 1968-1970; Assistant, Pathology, Univ. of Illinois, 1966-1968; Assistant Instructor, Cook County Hosp. School of Nursing, 1958-1959; Laboratory Teaching Asst., Loyola University, 1956-1958 and 1959-1962

Recent Relevant Publications

Robert E. Lee, Jr., M.D., Ph.D.

1. Lee, R.E., Jr., Hobart, E. and Aras, A.
   Inhibition of ATP-induced contraction of human myofibrils in vitro by antihuman myofibril rabbit serum. Lab. Invest., 22:504, 1970

2. Lee, R.E., Jr., Hobart, E., Aras, A. and Andresen, R.
   Electron microscopy of human myofibrils resistant to ATP-induced contraction following reaction with antiserum.
   Am. J. Path., 62:43a, 1971

3. Hobart, E.D., Lee, R.E., Jarosz, H. and Aras, A.
   A severe myopathy produced in guinea pigs with a diet containing lead and deficient in vitamin C.
   Fed. Proc., 30:522, 1971

4. Lee, R.E., Jr., Hughes, F.W., Aras, A. and Hass, G.M.
   Effects of extraction of myosin or actin on weight contraction and ultrastructure of myofibrils reacted with antimyofibril serum.
   Lab. Invest., 28:405-406, 1973

5. Hughes, W.F., Lee, R.E., Jr., Olson, R.H. and Hobart, E.D.
   Light and electron microscopic studies of a myopathy produced in guinea pigs fed a diet containing lead and deficient in vitamin C.
   Am. J. Path., 70:62a-63a, 1973

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
George M. Hass, M.D.		REDACTED
Reuben Eisenstein, M.D.		
Robert E. Lee, Jr., M.D.		
Technical		REDACTED
B. Zidonis (Histo. Tech.)		
M. Kedys (Res. Tech.)		
J. Dikinis (Chem. Tech.)		
R. Turner (Chief Tech.)		

Sub-Total for A 17500

B. Consumable supplies (by major categories)

Cost of 100 rabbits	750
Cost of diet cholesterol	500
Cost of laboratory reagents	1500
Cost of glassware, etc.	1200

Sub-Total for B 3950

C. Other expenses (itemize)

Animal Hospital care of a colony of 40 rabbits, each at 80 cents per day

Sub-Total for C 11680

Running Total of A + B + C 33130

D. Permanent equipment (itemize)

none

Sub-Total for D none

E. Indirect costs (15% of A+B+C) 4969

Total request 38099

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	18550	4345	12380	none	5291	40566
Year 3	19663	4779	13123	none	5635	43200

10035442S2

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Human and Experimental Atheroarteriosclerosis	USPHS HE-03215	$70,600	1971-1973
Miscellaneous Institutional		65,000	1972-1973, 1974-1975
Pathogenesis of Nicotine Induction of Calcific Athero-arteriosclerosis with Thromboarteritis in Rabbits	AMA Comm. Res. on Tobacco and Health	142,080	1967 - 1975
(see continuation sheet)			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Institutional		$65,000	1975-

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name George M. Hass, M.D.

Signature George M. Hass Date 06/03/74

Telephone 312 (Area Code) 942 (Number) 5219 (Extension)

Checks payable to

Rush Presbyterian-St. Luke's Med. Ctr.

Mailing address for checks

1753 W. Congress Parkway

Chicago, Illinois 60612

Responsible officer of institution

Typed Name William Hejna, M.D.

Title Vice Pres., Med. Affairs and Dean, Rush Medical College

Signature William Hejna

Telephone 312 (Area Code) 942 (Number) 5477 (Extension)

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16. (Continuation)

Currently Active

The Organization of Arterial Ground Substance	USPHS HL-14609	$89,547	1972-1975
A Proteinase Inhibitor in Vascular Walls	Chicago Ht. Assoc.	10,450	1973-1974
Schweppe Fellowship	Schweppe Fdn.	10,000	1973-1974
Institutional		7,500	1973-1974